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INFLUENCE OF OXYGEN TENSION ON MORPHOLOGIC VARIATIONS IN B. DIPHTHERIAE *

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Denny[1] showed by repeated and frequent examinations of pure cultures of the diphtheria bacillus, grown on Loeffler's blood serum at 36 C., that the young forms, C^2 and D^2, Wesbrook[2] types, gradually elongate, stain more and more irregularly, and tend to become club-shaped, or rarely branched, with age; further, that the rate at which these morphologic changes take place is delayed by a temperature below the optimum (19-21 C.), or, as Neisser also pointed out, the normal evolution may be suppressed by a temperature above the optimum (40 C.).

I was not acquainted with Denny's work when the following observations were made, so that they not only call attention to another physical factor in this variation, but also perhaps have some additional value in that they were not influenced by his work.

The culture used in this study was isolated from the throat of a typical case of diphtheria, on a slant of Loeffler's blood serum, kept aerobically at 37 C. The single colony subculture, after 24 hours' growth at 37 C. on the same medium, showed chiefly the A^1 type, along with C^1, and D^1, and intermediate types. The culture was plated out from salt solution emulsions by the successive stroke method, subcultures being made from well isolated colonies, 3 times. The final aerobic subculture on which the following observations were made still showed the barred types referred to above (Fig. 1).

The pathogenicity and ability to ferment carbohydrates was not tested until 9 months after isolation. For 5 months of the 9, the culture was kept at 5 C. on +1 human blood agar (anaerobic). When injected subcutaneously into guinea-pigs (0.5 c.c. of a 48-hour broth culture per 100 gm.), death occurred in 3-4 days with a typical serogelatinous edema. An anaerobic and an aerobic subculture on +0.5 Martin's medium containing human blood was tested: Slants of +0.5 Martin's medium (muscle sugar free) containing sterile ascites fluid

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[1] Jour. Med. Research, 1903, 9, p. 117.
[2] Tr. Assn. Am. Phys., 1900, 15, p. 198.

and sterile solutions of the following substances were inoculated, one set, from the aerobic that was kept under aerobic conditions; the other set, from the anaerobic which was kept under anaerobic conditions. The aerobic set showed no acid-production, but alkali-production, after 4 days at 37 C., in dextrose, levulose, galactose, lactose, saccharose, maltose, mannite, dextrin and starch, while the anaerobic culture showed marked acid-production from dextrose, only.

In the microscopic study of types, the smears were fixed by heat, after thorough drying high above the Bunsen flame, and stained for about 3 minutes with Loeffler's methylene blue.

When grown on parallel aerobic and anaerobic cultures on Loeffler's serum, the aerobic gave the foregoing types, whereas the anaerobic yielded almost entirely the D^2 type. Cross transfers were then made, aerobic from anaerobic and vice versa, on Loeffler's serum, and on + 0.5 and + 2.0 Martin's medium,[3] containing sterile human pleuritic fluid. The same inhibition in the evolution of the aerobic types was shown by all the anaerobic cultures made from the aerobic, while the aerobic cultures made from the anaerobic developed the D^1, C^1, and A^1 types.

If we determine the age of an organism, not by time alone, but by the time elapsing between youth, old age and death, then the lettering on Wesbrook's types should be reversed. The D^2 types are undoubtedly the youngest types, and if we can keep them in a state of suspended animation, they are still young even months later, compared with A^1 types, which have died in the interval. This was clearly shown by + 1 human blood-agar cultures kept for 5 months at 5 C. The 5-months-old anaerobic culture still showed the D^2 types (Fig. 2); these stained well and yielded luxuriant growth when transplanted aerobically and anaerobically, while the 5-months-old aerobic culture was dead.

When the D^2 types on an anaerobic + 1 human blood-agar culture were transferred to the same medium and incubated aerobically for 24 hours, the larger barred types developed, as shown in Figure 3.

In an anaerobic culture on Loeffler's blood serum, the growth was more luxuriant than in an anaerobic culture on + 1 human blood agar, where it was very delicate and the growth continued on Loeffler's serum at 37 C. for more than 24 hours; that is, a culture which in 24 hours showed chiefly the D^2 type, in 48 hours showed chiefly D^1 with C^1, and a few A^1 types.

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[3] Jour. Path. and Bacteriol., 1911, 15, p. 76.

The influence of oxygen on the evolution of the older types was shown in another way: The types present in 24-hour-old cultures on + 1 human blood agar were grown at the ordinary tension, at partial tension,[4] with the aid of B. subtilis, and anaerobically were recorded. The results of the aerobic and anaerobic growths corresponded with what has already been stated. In the partial tension culture inoculated from an aerobic slant, there was not much delay in the evolution of types, but in the partial tension culture inoculated from an anaerobic culture (D^2 types), very few of the organisms had gone beyond the D^1 type.

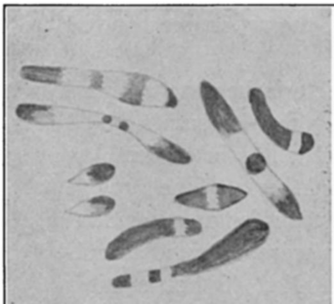
Fig. 1

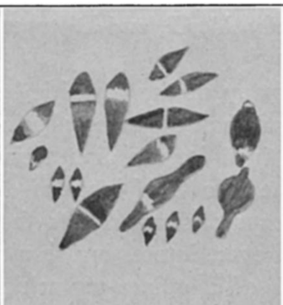
Fig. 2

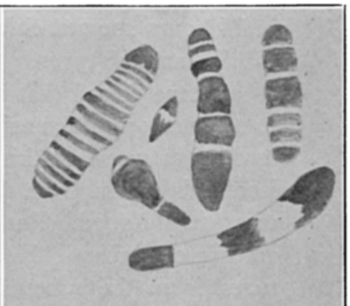
Fig. 3

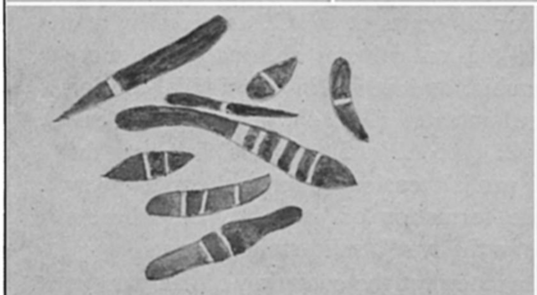
Fig. 4

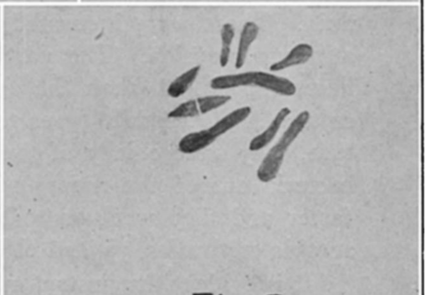
Fig. 5

Fig. 1.—Loeffler's serum, aerobic, 24 hours, 37 C. A^1, C^1, D^1 types, 4-6 microns × 0.8-0.5 micron; small Pyriform, about 0.9 micron by 0.5 micron (scale 1 micron = 0.2 inch).

Fig. 2.—+ 1 human blood agar slant, anaerobic, 24 hours, 37 C. D^2 and smaller types, 0.5-2.0 micron by 0.5-0.3 micron (scale 1 micron = 0.2 inch).

Fig. 3.—+ 1 human blood agar (inoculated with D^2 type from anaerobic culture), aerobic, 24 hours, 37 C; 1-6 microns by 0.8-1 micron (scale 1 micron = 0.2 inch).

Fig. 4.—+ 0.5 Martin's ascites agar, partial oxygen tension culture from subcutis of guinea-pig, 24 hours, 37 C. Longest forms 5-6 microns by 0.8 micron (scale 1 micron = 0.2 inch).

Fig. 5.—Culture similar to that shown in Fig. 4, but grown aerobically for 24 hours, 37 C, chiefly C^2 type. Longest forms 2-3 microns by 0.6 micron (scale 1 micron = 0.2 inch).

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[4] Wherry and Oliver: Jour. Infect. Dis., 1916, 19, p. 288.

As far as my work goes, it is evident that the oxygen supply has a more potent influence on the evolution of the larger types (involution forms, as some think) than has the composition of the medium, which however may influence the rate of multiplication as well as oxygen supply and temperature; that is, when grown on + 0.5 Martin's medium containing human blood, this culture showed equally luxuriant growth under aerobic and anaerobic conditions; but while the aerobic in 24 hours showed chiefly A^1, C^1, and D^1 types, the anaerobic showed D^2, E^2, and F^2 types.

I[5] have found that many of the parasitic bacteria become adapted to a lower oxygen tension than the atmospheric. Two attempts failed to demonstrate that this held for the diphtheria bacillus in cultures on Loeffler's serum from the throats of cases of diphtheria. The growth was equally good. Perhaps this was due to the transfer of superficial aerobic organisms. However, interesting results were obtained in cultures from the subcutis of a guinea-pig which died 48 hours after inoculation. A loopful of the serogelatinous exudate was rubbed over the surface of slants of + 0.5 Martin's medium, containing ascites fluid. These were incubated for 24 hours at 37 C., aerobically, and at partial tension. The partial-tension cultures showed much more luxuriant growth than the aerobic, and while in the partial-tension culture A^1, C^1, and D^1 types predominated (Fig. 4), in the aerobic culture, they were chiefly of the C^2 type (Fig. 5), that is, bringing the bacteria out of the tissues at partial tension enabled them to grow faster and they went on to the formation of barred types, whereas aerobic cultivation yielded chiefly the small, solid-staining type.

It is evident that in trying to interpret the evolution of the various morphologic types encountered in cultures from diphtheritic throats on Loeffler's serum, one must consider not only the variations in oxygen supply produced by associated organisms of various kinds, but also the possible influence of metabolic products set free in such mixed cultures. Denny has shown that granular forms (C, D) tend to appear in cultures mixed with staphylococci, or streptococci. It seems possible that the production of the granular types A, C, and D, may depend on the presence of certain organic acids as in the production of the granular types of tubercle bacilli.[6]

Wesbrook, Wilson, and McDaniel[2] clearly recognized and described the extreme variability of these so-called types, and never meant that they should be anything more than an aid to rapid diagnosis.

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[5] Wherry and Oliver: Jour. Infect. Dis., 1916, 19, p. 288; 1917, 20, p. 1.

[6] Wherry: Centralbl. f. Bacteriol., I, O., 1913, 70, p. 115.

CONCLUSIONS

The observations recorded appear to show that the occurrence of various morphologic types of the diphtheria bacillus in a mixed culture from the lesions of diphtheria is to be explained only when one appreciates the influence of oxygen on the rate of growth. They confirm the work of Denny in so far as they show that the normal evolution of the barred type begins with the D^2 type (or smaller), and develops successively the D^1, C^1, A^1, and sometimes the A type.

That the oxygen supply is not the only factor concerned is shown by the difference in the rate of growth and morphology in cultures grown anaerobically on media of different composition.

In mixed cultures from the throat, the morphology of diphtheria bacilli growing in association with other micro-organisms is probably modified by chemical products of growth, as well as by the reduced oxygen tension resulting from such association. Further, in making cultures from a diphtheritic membrane, one may transfer bacilli adapted to varying oxygen tensions; that is, if a bacillus adapted to a lower tension than the atmospheric is placed on the medium and grows unaccompanied by other species, its growth will be retarded and consequently its descendants will be of the solid-staining types.